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A two-step recognition of signal sequences determines the translocation efficiency of proteins

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The cytosolic and secreted, *N*-glycosylated, forms of plasminogen activator inhibitor-2 (PAI-2) are generated by facultative translocation. To study the molecular events that result in the bi-topological distribution of proteins, we determined *in vitro* the capacities of several signal sequences to bind the signal recognition particle (SRP) during targeting, and to promote vectorial transport of murine PAI-2 (mPAI-2). Interestingly, the six signal sequences we compared (mPAI-2 and three mutated derivatives thereof, ovalbumin and preprolactin) were found to have differential activities in the two events. For example, the mPAI-2 signal sequence first binds SRP with moderate efficiency and secondly promotes the vectorial transport of only a fraction of the SRP-bound nascent chains. Our results provide evidence that the translocation efficiency of proteins can be controlled by the recognition of their signal sequences at two steps: during SRP-mediated targeting and during formation of a committed translocation complex. This second recognition may occur at several time points during the insertion/translocation step. In conclusion, signal sequences have a more complex structure than previously anticipated, allowing for multiple and independent interactions with the translocation machinery.

Keywords: ovalbumin/secretion/serpin/signal peptide/SRP

Introduction

Secreted eukaryotic proteins are synthesized with an N-terminal targeting domain, the signal sequence, which promotes their translocation into the rough endoplasmic reticulum (RER) (Blobel and Dobberstein, 1975). Although signal sequences are extremely heterogeneous, a number of conserved features have been recognized and shown to be essential for protein export (von Heijne, 1985). These include a polar N-terminal region, which is usually positively charged, a central 7–15 amino acids long hydrophobic core and a polar C-terminal region where most signal sequences are cleaved by the signal peptidase complex. In spite of a very large sequence diversity, the functions of signal sequences have been maintained throughout evolution, as evidenced by the fact that many prokaryotic and eukaryotic signal sequences are functionally interchangeable, promoting protein export in a large variety of cells from many species (Gierasch, 1989).

The signal sequence is recognized, as it emerges from the ribosome, by a ribonucleoprotein complex, the signal recognition particle (SRP) (for recent reviews see Walter and Johnson, 1994; Lütcke, 1995). SRP targets the nascent chain–ribosome complex to the RER by interacting with the heterodimeric SRP receptor, thereby preventing the release of secreted proteins into the cytosol. The third function of SRP involves a translational control activity, which arrests or delays elongation of nascent chains until delivery to the RER membrane; translation arrest is strictly dependent on the recognition of signal sequences and is therefore specific for secreted proteins (reviewed in Strub *et al.*, 1993).

Biophysical and biochemical evidence indicates that nascent chains insert into and cross the RER membrane by passing through a proteinaceous channel which is sealed from the cytosol by ribosomes (Simon and Blobel, 1991; Crowley *et al.*, 1994). Several components of the translocation machinery, or translocon, have been identified and characterized (for recent reviews see Sanders and Sheckman, 1992; Gilmore, 1993). Both cross-linking and reconstitution experiments suggest that one or more component(s) of the translocon may interact functionally with signal sequences (Krieg *et al.*, 1989, Görlich *et al.*, 1992a,b; Hartmann *et al.*, 1993; High *et al.*, 1993; Mothes *et al.*, 1994; Nicchitta *et al.*, 1995). It was recently shown that the Sec61p complex is necessary and sufficient to allow SRP-independent translocation of preprolactin, thus providing evidence for a second recognition of signal sequences at the membrane (Jungnickel and Rapoport, 1995).

Plasminogen activator inhibitor-2 (PAI-2) is a member of the SERPIN family of serine–protease inhibitors (Huber and Carrell, 1989). The targets of PAI-2, like those of most other members of this family, are extracellular proteases (Vassalli *et al.*, 1991). However, two forms of PAI-2 are detected in cultures of mammalian cells: a secreted *N*-glycosylated form and a cytosolic non-glycosylated form. The relative amounts of these two forms can differ substantially (reviewed in Belin, 1993). For instance, most of the PAI-2 protein accumulates in the cytosol of undifferentiated U937 monocytic cells, while equivalent amounts of both forms are produced after differentiation of these cells (Wohlwend *et al.*, 1987). The synthesis of both forms of human PAI-2 (hPAI-2) occurs on the same mRNA, and starts at the same AUG codon. The bi-topological distribution of this protein has therefore been proposed to result from a facultative translocation event (Belin *et al.*, 1989).

PAI-2 belongs to a sub-group of the SERPINs, which includes ovalbumin, a secreted protein, as well as elastase

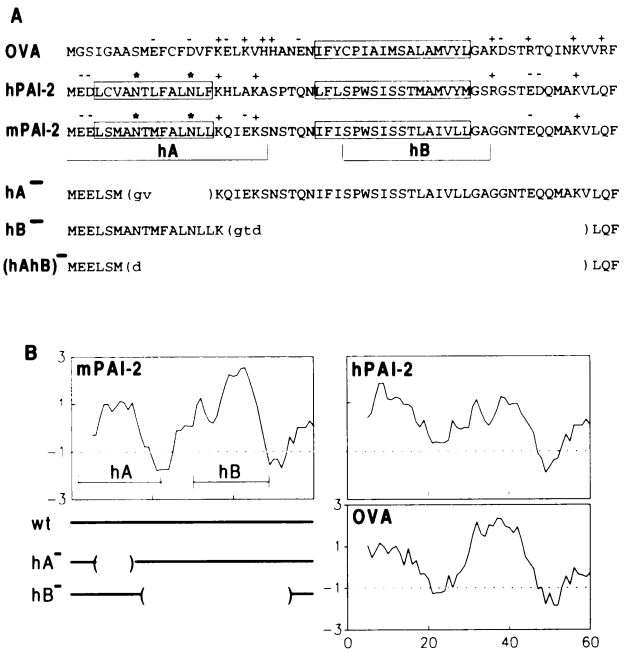

Fig. 1. The N-terminal region of PAI-2. (**A**) Alignment of the N-terminal 60 amino acids of hen ovalbumin (SwissProt AN: P01014) and of human and murine PAI-2 (SwissProt AN: P05120 and P12388 respectively) are shown in the one-letter code. The PAI-2 proteins have an overall sequence conservation of 76%. The conserved Asn residues are highlighted (*) and the charged residues are marked (+/–). The hydrophobic elements are boxed. The position of the two α-helices are indicated (Huber and Carrell, 1989). The N-terminal amino acid sequences encoded by the truncated mPAI-2 proteins are shown below; amino acids added during cloning are shown in lower case. (**B**) Hydropathy plots of the N-terminal 60 amino acids of mPAI-2, hPAI-2 and ovalbumin. The sequences were analysed with a hydrophobic scale (Kyte and Doolittle, 1982), using an interval of nine amino acids. The hydrophobic portions of the hA and hB domains of PAI-2 are centred at amino acids 11 and 36 respectively. The hA domain of ovalbumin is charged and its signal sequence has been located to the hB domain (Tabe *et al.*, 1984).

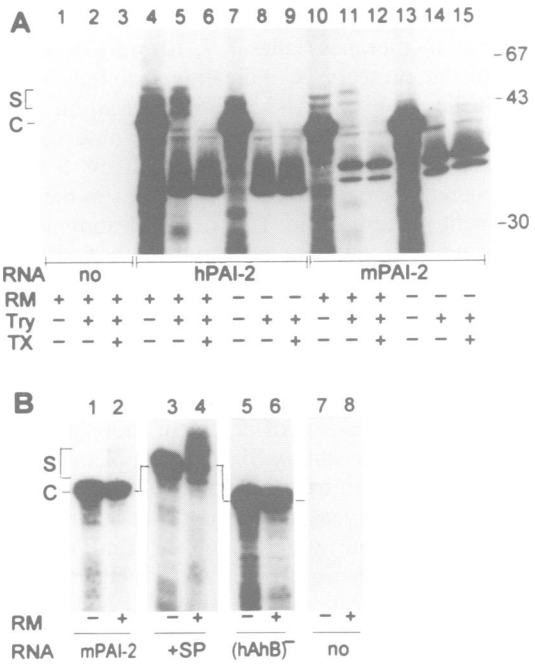

Fig. 2. *In vitro* translocation of PAI-2 in the presence of microsomal membranes. (**A**) *In vitro* synthesized human (lanes 4–9) and murine (lanes 10–15) PAI-2 mRNAs were translated in a wheat-germ extract in the presence (lanes 4–6 and 10–12) or in the absence (lanes 7–9 and 13–15) of canine microsomal membranes (RM). After 90 min at 26°C, translation was terminated by the addition of 100 μg/ml pancreatic RNase. Aliquots were digested for 30 min at 0°C with 1 mg/ml trypsin (Try) in the absence (lanes 2, 5, 8, 11 and 14) or in the presence (lanes 3, 6, 9, 12 and 15) of 0.4% TX-100 (TX). The samples were analysed by SDS–PAGE followed by fluorography. Control translations without added mRNA are shown in lanes 1–3. The M_r 33 000–37 000 digestion products (lanes 5, 6, 8, 9, 11, 12, 14 and 15) are trypsin degradation intermediates of hPAI-2 and mPAI-2; the M_r 32 000 product in lane 4 is probably due to the presence of contaminating proteases in the RM. The positions of marker proteins electrophoresed in a parallel lane are indicated on the right side. (**B**) Synthetic mRNAs were translated in the presence (+) or in the absence (–) of microsomal membranes (RM). Lanes 1 and 2: complete mPAI-2; lanes 3 and 4: fusion of the yeast prepro-α-factor signal sequence to the N-terminus of PAI-2; lanes 5 and 6: mPAI-2 lacking the hA and the hB domains $(hAhB)^-$; lanes 7 and 8: control translations without added mRNA. C: cytosolic PAI-2; S: *N*-glycosylated translocated PAI-2.

and thrombin inhibitors that have only been detected in the cytosol (Belin, 1993; Remold-O'Donnell, 1993). PAI-2, like ovalbumin, has only the minimal size to fold as a SERPIN (Huber and Carrell, 1989) and both proteins are secreted without cleavage of their signal sequences (Tabe *et al.*, 1984; Ye *et al.*, 1988). Two regions have been proposed to promote PAI-2 translocation (Ye *et al.*, 1988; Belin *et al.*, 1989): an N-terminal hydrophobic domain within the first α-helix and an internal hydrophobic domain spanning the second α-helix [hA and hB domains (Huber and Carrell, 1989); Figure 1]. The *in vitro* translocation efficiency of mutated hPAI-2 proteins, which either lacked internal amino acids or had amino acid substitutions, suggested that both domains cooperate to promote translocation of hPAI-2 and that its signal sequence 'is inefficient by design rather than necessity' (von Heijne *et al.*, 1991).

We have studied the translocation of murine PAI-2 (mPAI-2) *in vitro*. We found that two events determine the low efficiency of mPAI-2 translocation. First, the recognition of the internal mPAI-2 signal sequence by SRP was inefficient. Secondly, only a small fraction of the SRP-bound molecules were actually translocated. Thus, a second recognition of signal sequences at the membrane also determines the overall efficiency of translocation. This notion was further supported by the analysis of the capacity of several other signal sequences to bind SRP and to promote protein translocation. Finally, membrane flotation experiments showed that the second recognition of signal sequences can occur both before and after the formation of stable nascent chain–ribosome–membrane complexes.

Results

Low translocation efficiency of mPAI-2 reflects an intrinsic propriety of its signal sequence

We have previously shown that translocation of *in vitro* synthesized hPAI-2 into canine microsomal membranes is inefficient (Belin *et al.*, 1989). We first compared the translocation efficiency of mPAI-2 with that of its human counterpart; the N-terminal sequence of both proteins is shown in Figure 1. The major translation products of the human and murine synthetic PAI-2 mRNAs (Figure 2A),

observed both in the absence and in the presence of microsomal membranes (lanes 4, 7, 10 and 13), were fully susceptible to proteolysis, indicating that they represent the cytosolic form of PAI-2. Minor translation products with slower mobilities were exclusively observed in the presence of microsomal membranes (lanes 4 and 10). These proteins were resistant to proteolysis in the absence of detergent (lanes 5 and 11), demonstrating that they were located in a membrane-enclosed compartment. These products represent partially *N*-glycosylated PAI-2 proteins, in agreement with the presence of four and three Asn-X-Ser/Thr sites in mPAI-2 and hPAI-2 respectively. Under the same conditions, urokinase-type PA, another secreted protein, was efficiently translocated and protected from proteolysis in the absence of detergent (not shown).

To exclude the possibility that internal elements within mPAI-2 interfere with translocation, we analysed a fusion protein in which the yeast prepro-α-factor signal sequence precedes the complete coding sequence of mPAI-2. Translocation of the chimeric protein was efficient, as judged by the relative intensities of translocated, *N*-glycosylated and unmodified proteins (Figure 2B, lanes 3 and 4); similar results have been obtained with hPAI-2 (Steven *et al.*, 1991; von Heijne *et al.*, 1991). We then constructed a large truncation which removes both mildly hydrophobic domains in the N-terminal region but still retains three of the four *N*-glycosylation sites of mPAI-2 [(hAhB)$^-$; Figure 1A]. Translation of this mRNA resulted in the synthesis of only one product with and without microsomal membranes (Figure 2B, lanes 5 and 6), indicating that this protein is not translocated. Thus, the signal sequence of mPAI-2 is located within the first 57 amino acids of the protein and promotes translocation of only a small fraction of the molecules. Inefficient translocation of PAI-2 *in vitro* is an intrinsic property that has been conserved between primates and rodents.

Internal signal sequence of mPAI-2 is recognized twice during translocation

Protein translocation into the RER has commonly been divided into two steps: the SRP-mediated targeting step, during which the nascent chain–ribosome complex is delivered to the RER, and the insertion/translocation step, during which the polypeptide chain is moved across the membrane. To unravel the mechanism of the inefficient translocation of mPAI-2, we decided to examine each step individually.

The binding of SRP to the signal sequence of a nascent chain in the absence of microsomal membranes specifically diminishes its rate of elongation, leading to a complete arrest or to a delay in the accumulation of full-length protein. Although the exact mechanism by which SRP affects elongation has not been elucidated, it was shown that ribosomes pause naturally at certain sites in nascent chains, and that the delay in protein synthesis caused by SRP is due to enhanced pausing at these sites (Wolin and Walter, 1988, 1989). While the extent of pausing appears to be determined by the nature of the natural pause sites, elongation arrest, when detected, is strictly dependent on signal sequence recognition by SRP and therefore provides an independent and quantitative means of measuring this interaction.

We first assayed whether SRP had an effect on the

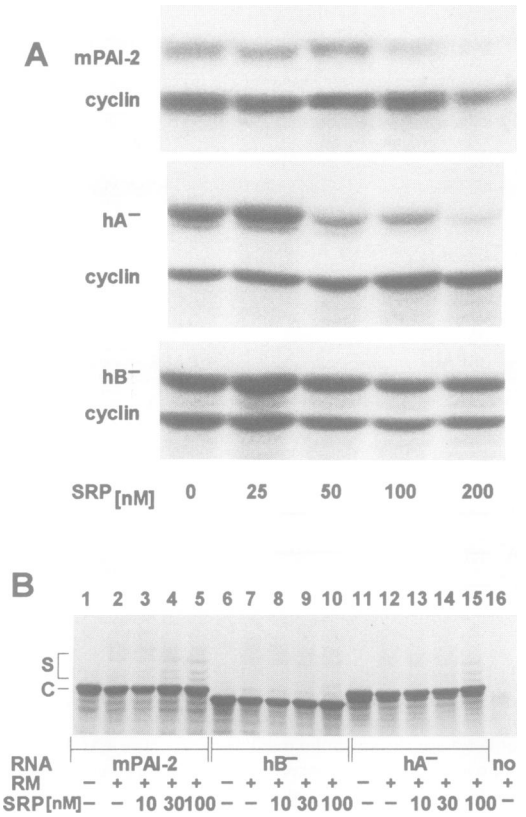

Fig. 3. SRP-binding and vectorial transport promoted by the mPAI-2 internal signal sequence. (**A**) mPAI-2 and cyclin mRNAs were translated for 20 min in the presence of the indicated concentrations of purified SRP, but in the absence of microsomal membranes. Cyclin, a cytosolic protein, served as an internal standard for translation efficiency. The extent of the N-terminal truncations in the hA$^-$ and hB$^-$ variants is shown in Figure 1. (**B**) The synthetic mRNAs were translated in the absence (lanes 1, 6 and 11) or presence (lanes 2–5, 7–10 and 12–15) of microsomal membranes (RM); endogenous SRP concentration was ~10 nM. Purified SRP was added at the concentrations indicated. Lanes 1–5: complete mPAI-2; lanes 6–10: mPAI-2 containing only the hA domain (hB$^-$); lanes 11–15: mPAI-2 containing only the hB domain (hA$^-$); lane 16: control translation without added mRNA. C: cytosolic PAI-2; S: *N*-glycosylated translocated PAI-2.

accumulation of full-length mPAI-2 protein. Indeed, we found that the synthesis of mPAI-2 was specifically inhibited in the presence of increasing concentrations of SRP, whereas the synthesis of a sea urchin cyclin, a control cytosolic protein (Murray *et al.*, 1989), was not affected by SRP (Figure 3A). This result demonstrated that the signal sequence of mPAI-2 can bind SRP and promote elongation arrest. At very high SRP concentrations, elongation of >50% of the nascent chains was arrested. Thus, these chains remained stably bound to SRP throughout the incubation. When compared with preprolactin (Figure 4A), a ten times higher SRP concentration was required to arrest the elongation of 50% of the mPAI-2 nascent chains. In experiments described below, we found that other signal sequences can promote a complete arrest in the elongation of mPAI-2 nascent chains, indicating that the incomplete arrest observed here reflects a weaker interaction between the mPAI-2 signal sequence and SRP.

The individual contribution of each N-terminal domain

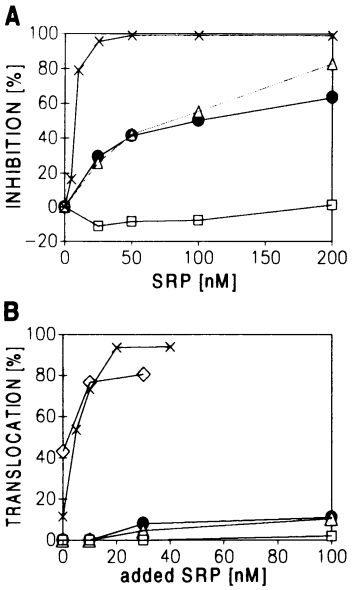

Fig. 4. A quantitative comparison of SRP binding and translocation efficiency mediated by the mPAI-2 internal signal sequence. (**A**) The amount of mPAI-2 synthesized at each SRP concentration (Figure 3A) was normalized to that of cyclin after quantification of the gels with a PhosphorImager. The data represent the average of two complete experiments. (●) complete mPAI-2; (△) mPAI-2 containing only the hB domain (hA^-); (□) mPAI-2 containing only the hA domain (hB^-); (×) preprolactin. (**B**) The efficiency of translocation was calculated by measuring the amounts of *N*-glycosylated and non-glycosylated products (Figure 3B), after densitometric analysis of the autoradiograms. The data represent the average of three complete experiments. (●) complete mPAI-2; (△) mPAI-2 containing only the hB domain (hA^-); (□) mPAI-2 containing only the hA domain (hB^-); (×) preprolactin; (◇) fusion of the yeast prepro-α-factor signal sequence to the N-terminus of PAI-2.

of mPAI-2 was then examined using truncated proteins that lack either the hA or the hB domain (Figure 1). The hB domain alone was recognized by SRP with the same apparent efficiency as the complete N-terminal region (Figures 3A and 4A), indicating that the hA domain has no detectable negative effect on hB recognition by SRP. In contrast, the synthesis of a protein lacking the hB domain was not reduced even in the presence of 200 nM SRP, showing that this protein was not detectably recognized by SRP. Thus, the signal sequence of mPAI-2 appears to be entirely located within the internal hB domain.

These results suggested that the inefficient translocation of mPAI-2 observed above (Figure 2) is due to a low SRP concentration in our microsomal membranes. Indeed, quantitation of the SRP 7S RNA showed that its concentration in the translocation assay was ~10 nM (not shown). We therefore complemented the translocation assay with purified SRP, and investigated its effect on the translocation efficiency of mPAI-2 (Figures 3B and 4B). Exogenous SRP significantly improved mPAI-2 translocation, as judged by the increased accumulation of *N*-glycosylated products (Figure 3B, lanes 3–5). As expected from the elongation arrest experiments, this effect was only detected when the concentration of added SRP was >10 nM. However, even at 100 nM of added SRP, only 10–13% of mPAI-2 was translocated (Figure 4B). Similarly, translocation of hPAI-2 was only slightly increased by added SRP (not shown). This effect was specific for PAI-2, since the fusion protein in which the yeast prepro-α-factor signal sequence precedes the complete coding sequence of mPAI-2 was translocated with an efficiency of 76–81% at 10 or 30 nM of added SRP (Figure 4B). Since the elongation of ~50% of the mPAI-2 nascent chains was arrested by SRP at these concentrations (Figure 4A), the level of translocation was unexpectedly low. It indicated that a large fraction of the SRP-bound nascent chains completed their synthesis in the cytosol, even in the presence of microsomes. These results suggest that the efficiency of mPAI-2 translocation is determined not only by the recognition of its signal sequence during the SRP-dependent targeting step, but also during the insertion/translocation step. This interpretation is validated by experiments showing that elongation arrest of nascent chains can be released upon addition of membranes (see below).

We examined whether translocation promoted by each N-terminal domain of mPAI-2 responds to the addition of exogenous SRP (Figures 3B and 4B). Translocation mediated by the hB domain was increased in the presence of exogenously added SRP to a similar extent as that observed with the complete protein (Figure 3B, lanes 13–15). In contrast, translocation mediated by the hA domain remained very low even in the presence of exogenous SRP (Figure 3B, lanes 8–10), as expected from the undetectable recognition of this domain by SRP. The truncated mPAI-2 protein missing both the hA and hB domains was not translocated even at 100 nM SRP (not shown), consistent with the previous finding that the export information of mPAI-2 is located within the N-terminal region.

SRP recognition and translocation mediated by ovalbumin signal sequence

Ovalbumin, another closely related member of the SERPIN family, also has an internal signal sequence localized to the hB domain (Tabe *et al.*, 1984). It has been reported that ovalbumin translocation is much less efficient *in vitro* than *in vivo* (Palmiter *et al.*, 1980). To expand our initial observation that overall translocation efficiency can be determined not only at the SRP-mediated targeting step but also at the insertion/translocation step, we decided to study translocation promoted by the ovalbumin signal sequence. To this end, we have replaced the complete N-terminal region of mPAI-2 by the equivalent region of ovalbumin (Figure 1), and determined the efficiency of translocation of this chimeric protein (OVA-PAI2) at each step individually.

We took advantage of the elongation arrest assay to study the binding of the ovalbumin signal sequence to SRP. As shown in Figure 5A, the synthesis of the OVA-PAI2 protein was efficiently inhibited by SRP, and a 50% elongation arrest was already achieved at <10 nM SRP. Since the translation pause sites of the OVA-PAI2 protein are beyond the signal sequence and are therefore the same as those of mPAI-2, these results indicate that the signal sequence of ovalbumin is recognized significantly better by SRP than its mPAI-2 counterpart. However, the overall translocation efficiency of the OVA-PAI2 protein was quite low at all SRP concentrations assayed (Figure 5B). For example, at 100 nM SRP, a concentration at which most nascent chains are expected to be bound to SRP, only 15–20% of the protein was translocated. Thus, the efficiency of translocation mediated by the internal signal

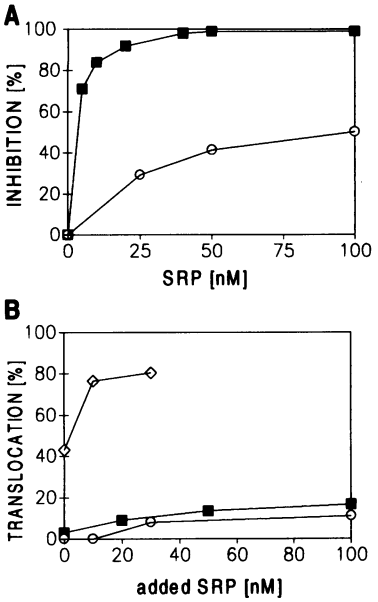

Fig. 5. A quantitative comparison of SRP binding and translocation efficiency mediated by the ovalbumin signal sequence. (**A**) The amount of ovalbumin synthesized at each SRP concentration was normalized to that of cyclin after quantification of the gels with a PhosphorImager. The data represent the average of two experiments. (■) ovalbumin; (○) mPAI-2 (the data are from Figure 4A). (**B**) The efficiency of translocation was calculated by measuring the amounts of *N*-glycosylated and non-glycosylated products, after densitometric analysis of the autoradiograms. The data represent the average of three experiments. (■) ovalbumin; (○) mPAI-2 (the data are from Figure 4B); (◇) fusion of the yeast prepro-α-factor signal sequence to the N-terminus of PAI-2.

sequence of ovalbumin appears to be primarily controlled during the insertion/translocation step.

Two conserved Asn residues in hA domain have different effects on SRP recognition and translocation

The hA domain of mPAI-2 has a hydrophobic core which could promote translocation, but it was not recognized by SRP as a signal sequence (Figures 3 and 4). It contains two features that may explain its lack of signal sequence activity: (i) the hydrophobic region is interrupted by two conserved Asn residues at positions 8 and 14; (ii) the N-terminus contains two negatively charged residues (Figure 1). To investigate the relative contribution of each of these four unusual amino acids, we have constructed a set of mutated hA domains, and measured their signal sequence activity (Figure 6).

Translocation of mPAI-2 mediated by the hA domain alone and by two mutated hA domains containing one or two N-terminal Lys residues is shown in Figure 6A. When either one or both N-terminal Glu residues were changed to Lys, translocation efficiency was only slightly increased, as <5% of the proteins were *N*-glycosylated. The three proteins analysed in Figure 6A contain two Asn residues in the hA domain. When one or the other Asn residue was replaced by Ile, translocation was markedly more efficient (Figure 6B and C). Replacement of both Asn residues resulted in translocation of 50–60% of the protein (Figure 6B, lanes 4 and 10; and C, lane 2). Thus, the lack of signal sequence activity of the hA domain of mPAI-2 is mainly caused by the presence of two Asn residues which interrupt its hydrophobic core, and this domain can be converted into an efficient signal sequence by two amino acid substitutions.

The effect of N-terminal substitutions introduced in hA domains which have one of the Asn residues replaced by Ile (Figure 6B and C) shows that this domain became a more efficient signal sequence as the N-terminal charge increased. For instance, the Asn8–Ile14 variants were translocated with an efficiency of 9% in the Glu2Glu3 background (Figure 6B, lane 2), of 21% in the Lys2Glu3 background (Figure 6C, lane 4) and of 53% in the Lys2Lys3 background (Figure 6B, lane 8). Thus, and in agreement with previous results (Szczesna-Skorupa and Kemper, 1989), the inhibitory effect of the negatively charged Glu residues on translocation became significant only when the core of the signal sequence had an intermediate hydrophobicity.

We have shown that the inefficient translocation mediated by the hB domain of mPAI-2 results from two events: a weak recognition by SRP and the translocation of only a fraction of the SRP-bound nascent chains. It was therefore of interest to determine whether the detrimental effect of the two Asn residues in the hA domain is exerted at the targeting and/or the insertion/translocation step. As shown in Figure 7A, elongation of the Asn8–Ile14 signal sequence variant was only moderately inhibited by SRP, with a 50% elongation arrest achieved at ~120 nM SRP. In contrast, the Ile8–Asn14 signal sequence variant promoted a 50% elongation arrest at an SRP concentration of 15 nM, indicating that it was much better recognized by SRP than the hB domain and the Asn8–Ile14 hA domain variant. Finally, when both Asn residues were replaced by Ile, the hA domain promoted an SRP-dependent elongation arrest as efficiently as the signal sequence of preprolactin (Figure 7A).

We then compared the effect of exogenous SRP on translocation mediated by three variants of the hA domain in which either one or both Asn residues had been replaced by Ile residues (Figure 7B). As shown above (Figure 6B, lane 4), an hA domain in which both Asn were replaced by Ile was already efficiently translocated in the absence of added SRP, and ~70% of the protein was translocated with 40 nM added SRP. Translocation mediated by the Asn8–Ile14 signal sequence variant was markedly improved by the addition of SRP and reached ~30% at an SRP concentration of 100 nM. Thus, this hA domain variant showed a translocation efficiency similar to that predicted from the elongation arrest experiment, since almost all the SRP-bound nascent chains were translocated. In contrast, translocation mediated by the Ile8–Asn14 signal sequence variant was only 40% efficient, even at SRP concentrations that allowed nearly complete SRP binding in the translation arrest experiments. These results further demonstrate that the activity of a signal sequence can be determined not only at the SRP-dependent targeting step, but also at the insertion/translocation step.

Signal sequence recognition during the insertion/translocation step

The model described above is based on the assumption that the elongation arrest assay demonstrates the association of SRP with nascent chains, and that only a fraction of the SRP-bound nascent chains get translocated. This implies

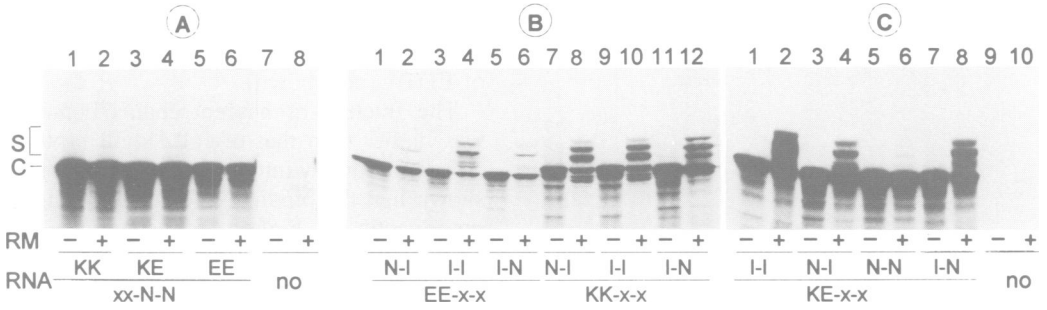

Fig. 6. Translocation promoted by hA domain variants. The mRNAs were translated in the absence (–) or in the presence (+) of microsomal membranes (RM). All the mutated proteins have the hA domain fused to Leu58 of mPAI-2, and therefore lack the hB domain. The sequence of the four amino acids that are changed in the mutated proteins is indicated with the one-letter amino acid code: K = Lys, E = Glu, N = Asn and I = Ile. X is K or E (**A**), and I or N (**B** and **C**). C: cytosolic PAI-2; S: *N*-glycosylated translocated PAI-2.

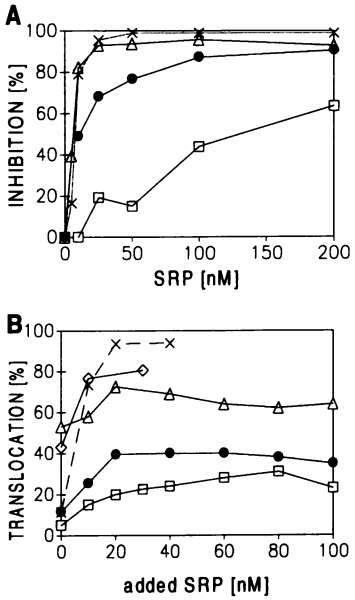

Fig. 7. Differential effects of two conserved Asn residues in the hA domain on SRP-binding and on translocation. The three mutated hA domains had Asn8, Asn14 or both residues replaced by Ile residues; all three proteins contain two N-terminal Glu residues and lacked the hB domain. (□) Asn8–Ile14; (●) Ile8–Asn14; (△) Ile8–Ile14; (×) preprolactin. The data represent the average of two complete experiments. (**A**) The mRNAs were translated for 20 min in the presence of the indicated concentrations of purified SRP, but in the absence of microsomal membranes. The relative amount of mPAI-2 variants synthesized at each SRP concentration was normalized to that of cyclin and compared with that synthesized in the absence of SRP. (**B**) The mRNAs were translated in the presence of microsomal membranes, and purified SRP was added at the concentrations indicated. The efficiency of translocation was calculated by measuring the amounts of *N*-glycosylated and non-glycosylated products, after densitometric analysis of the autoradiograms. (◇) fusion of the yeast prepro-α-factor signal sequence to the N-terminus of PAI-2.

that the arrested protein fragments are indeed elongated upon interaction with microsomal membranes. We therefore decided to visualize the arrested fragments and to determine their fate with a two-step translocation assay (Figure 8).

The translation of mPAI-2 and of OVA-PAI2 was allowed to occur for 20 min in the absence or in the presence of SRP (Figure 8A and B). In the presence of SRP, a series of small protein fragments were visualized, and the amount of full-length protein was significantly reduced. In agreement with the data shown in Figures 4A and 5A, the specific inhibition was 85 and 50% for OVA-PAI2 and mPAI-2 respectively. The size range of the arrested fragments was similar to that observed with preprolactin (Figure 8C). Upon addition of microsomal membranes, and in the presence of a cap analogue to inhibit new rounds of translation initiation, the amount of small protein fragments was much reduced, and there was a corresponding increase in the synthesis of full-length proteins (Figure 8A and B). In addition, *N*-glycosylated OVA-PAI2 products were now detected. We did not expect to detect the N-glycosylated forms of mPAI-2, since 50% of the nascent chains were already terminated before the addition of microsomal membranes; in addition, translocation of OVA-PAI2 was somewhat more efficient (Figure 5). Control experiments with preprolactin showed that 80–90% of the protein was converted to the mature form by cleavage of its signal sequence (Figure 8D). These data indicate that SRP-bound nascent chains can be elongated upon interaction with the translocation machinery, and that the decision to transport the protein vectorially into the microsomal lumen or to release it in the cytoplasm occurs during the insertion/translocation step.

The insertion of SRP-bound nascent chains into the translocation machinery results in the formation of stable complexes between ribosomes and microsomal membranes, that are resistant to high salt and EDTA treatment (Gilmore and Blobel, 1985; Connolly and Gilmore, 1986). To determine whether the fate of the mPAI-2 nascent chains is determined before or after the formation of these stable complexes, we have translated short truncated transcripts in the presence of SRP and microsomal membranes, and separated free and membrane-bound complexes by flotation in sucrose gradients (Figure 9). The nascent chains analysed here were 143 amino acids long. At an SRP concentration which can promote a 50% elongation arrest, ~20% of the mPAI-2 nascent chains were recovered as high salt-resistant membrane-bound complexes (Figure 9A); when the hB domain was deleted, <2% of the nascent chains were membrane-bound (not shown). A densitometric profile of the free nascent chains showed a family of products with increasing sizes (Figure 9B). The profile of the membrane bound nascent chains was different, revealing similar amounts of larger *N*-glycosylated products and of unmodified nascent chains. At an SRP concentration which can promote a >90% elongation arrest, ~2/3 of the OVA-PAI2 nascent chains were recovered as membrane-bound complexes

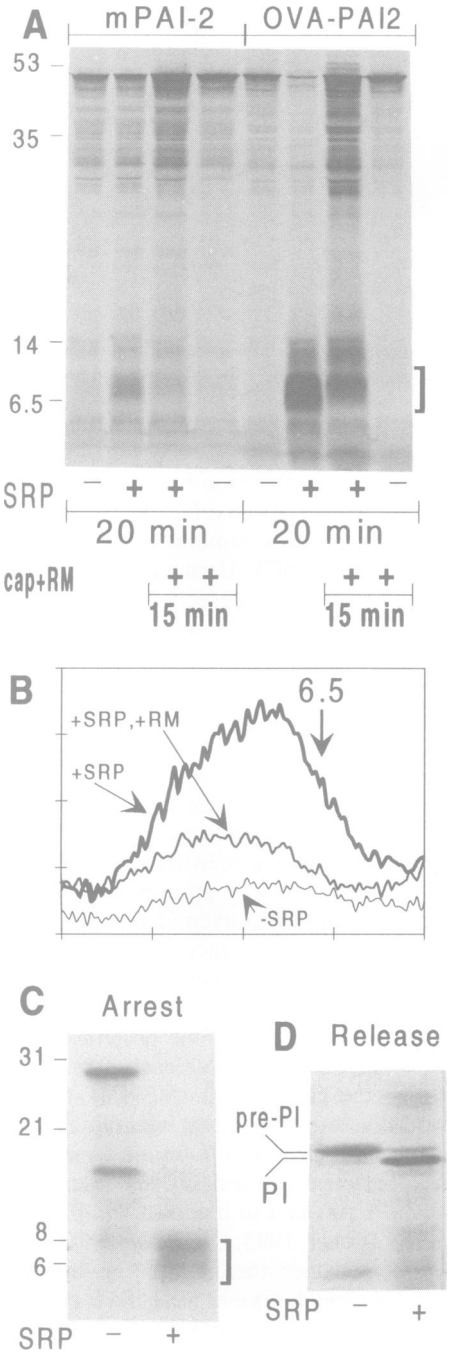

Fig. 8. Release of the elongation arrest upon addition of microsomal membranes. (**A**) The mPAI-2 and OVA-PAI2 mRNAs were translated for 20 min in the absence or in the presence of 100 nM SRP. After removing half of each sample, the cap analogue m^7GpppG was added to 1 mM to inhibit further initiation. SRP-depleted microsomal membranes were added to 1 Eq/10 µl, and the incubations were continued for 15 min. The arrested fragments, in the size range of M_r 6 000–12 000, are indicated by a bracket. The autoradiogram was overexposed to visualize the *N*-glycosylated forms of OVA-PAI2. Marker proteins are indicated on the left side. (**B**) Densitometric profiles of the mPAI-2 translation products in the M_r 5 000–12 000 size range from the first three lanes in (A); quantitation of the results showed that elongation of 75% (mPAI-2) and 60% (OVA-PAI2) of the arrested fragments resumed upon addition of membranes. (**C**) Control experiment with preprolactin in the absence of membranes. (**D**) Release of the elongation arrest upon addition of membranes. The samples in (A) and (C) were electrophoresed in 15 and 12% polyacrylamide gels respectively.

(Figure 9C). For both proteins, identical results were obtained in flotation experiments performed in the presence of EDTA (not shown).

The fraction of nascent chains bound to membranes was higher than that of full-length proteins translocated co-translationally into microsomal membranes. It has been shown that the binding of SRP to the signal sequence of preprolactin is highest for short nascent chains and decreases during elongation, presumably because the signal sequence becomes inaccessible (Siegel and Walter, 1988a). Based on this observation, the increased binding of nascent chains to microsomal membranes could reflect, at least partially, an improved binding of SRP to nascent chains which cannot be elongated beyond position 143. However, this interpretation appears unlikely in the case of OVA-PAI2, since elongation arrest on full-length mRNA was essentially complete at the SRP concentration used. Thus, these results suggest that the second recognition of signal sequences at the insertion/translocation step can occur both before and after the formation of stable nascent chain–ribosome–membrane complexes. If this is the case, some nascent chains can be stably bound to the membranes, but they will not be vectorially transported into the lumen.

Discussion

We have previously proposed that the bi-topological distribution of PAI-2 between the cytosol and the extracellular space results from facultative translocation of the protein into the RER (Belin *et al.*, 1989). In this study, we have analysed the capacity of several signal sequences to bind SRP during the targeting step, and to promote vectorial transport of mPAI-2 during the insertion/translocation step. Our results revealed a remarkable difference between the efficiencies of the two steps for several signal sequences. These differences can be explained by a model in which signal sequences are recognized first by SRP during targeting, and then, in a second independent event, at the RER membrane (Figure 10). Thus, the bi-topological distribution of PAI-2 can be explained by a combination of a moderate efficiency in the first recognition event and a low efficiency in the second one.

Two mildly hydrophobic domains in the N-terminal region of PAI-2 have been proposed to function as signal sequences, the hA and the hB domain (Ye *et al.*, 1988; Belin *et al.*, 1989; von Heijne *et al.*, 1991). Our results show that the hB domain appears to be the only secretion-promoting determinant of mPAI-2. Likewise, the signal sequence of hen ovalbumin was also localized to the hB domain (Tabe *et al.*, 1984). In contrast, the translocation efficiency of hPAI-2 mediated by the hA or the hB domain was lower than that observed with the complete N-terminal region, suggesting that both domains cooperate to promote translocation (von Heijne *et al.*, 1991). The difference between hPAI-2 and mPAI-2 may be explained by the more hydrophobic character of the mPAI-2 hB domain (Figure 1B).

The hA domain of mPAI-2 lacks signal sequence activity, but it can be converted into an efficient signal sequence by a limited number of amino acid substitutions. This analysis illustrates the different negative effects of two conserved Asn residues on translocation. An Ile to Asn substitution in the signal peptide of factor IX results

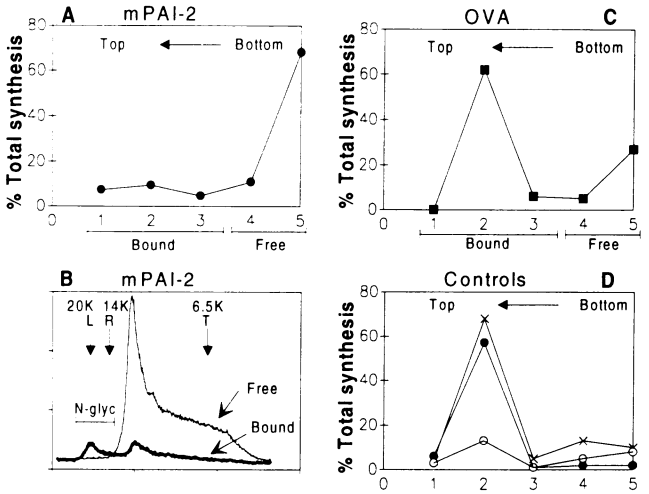

Fig. 9. Stable association of mPAI-2 and OVA-PAI2 nascent chains with microsomal membranes. (**A** and **B**) Free- and membrane-bound nascent chain–ribosome complexes were separated by flotation in sucrose gradients containing 0.5 M KOAc. The SRP concentration was 100 nM. The nascent chains in each fraction were resolved in 15% polyacrylamide gels, and quantitated with a PhosphorImager. (**C**) Densitometric profiles of the mPAI-2 free- and membrane-bound nascent chains. Marker proteins, lysozyme, RNase A and Aprotinin, are indicated. (**D**) Control experiments performed with 20 nM SRP and microsomal membranes. (×) flotation of preprolactin nascent chains (86 amino acids long); 77% of the nascent chains were recovered in the upper part of the gradient. Flotation of the full-length Ile8–Ile14 hA domain variant (Figure 7): the cytosolic (○) and *N*-glycosylated (●) forms were separated in 8% polyacrylamide gels. The *N*-glycosylated forms, which represented 69% of the total protein, were quantitatively recovered with the membranes. For the unmodified protein, 50% was not associated with membranes. The remaining portion could represent a small fraction of translocated protein which has not been *N*-glycosylated. This would account for the small difference in calculated translocation efficiencies between preprolactin and the chimeric protein with the yeast prepro-α-factor signal sequence fused to the N-terminus of PAI-2 (Figure 4B).

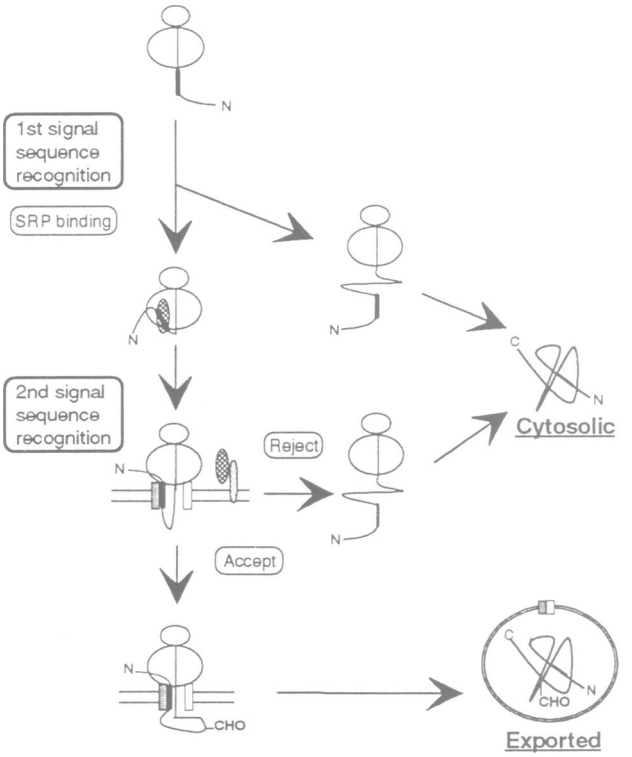

Fig. 10. Two signal sequence recognition events determine the translocation efficiency of proteins. During the first signal sequence (solid bar) recognition, the efficiency of SRP (cross-hatched oval) binding will determine the number of nascent chains that are targeted to the SRP-receptor (stippled oval) in the membrane. Elongation of the remaining chains is completed in the cytosol. The second recognition of signal sequences is mediated by component(s) of the translocon (hatched and empty bar). It may occur at several time points during the insertion/translocation step. The criteria that determine signal sequence recognition at each step can be different. *N*-glycosylation of the nascent chain (CHO) may contribute to the vectorial transport of proteins.

in a severe secretion defect and haemophilia B (Giannelli *et al.*, 1994), while a Thr to Asn substitution in the signal sequence of the yeast prepro-α-factor has only a small effect on translocation (Allison and Young, 1989). Taken together, these results demonstrate that the presence of a single Asn residue in the hydrophobic core of signal sequences can exert different, context-dependent effects.

In our functional dissection of signal sequences, we found three examples in which the translocation efficiency was accounted for by the capacity of the signal sequence to bind SRP: preprolactin, a widely used substrate in translocation assays, and the Ile8–Ile14 hA domain variant, were efficiently recognized by SRP and translocated; the moderate translocation efficiency of the Asn8–Ile14 hA domain variant is accounted for by an intermediate efficiency of SRP binding. However, three other signal sequences, the mPAI-2 and ovalbumin hB domains, and the Ile8–Asn14 hA domain variant, did not promote translocation to the extent expected from their SRP-binding efficiencies. This implies that SRP-bound nascent chains are not necessarily vectorially transported into the microsomal lumen. They can be released at stage(s) which would allow their synthesis to be completed in the cytosol. Thus, signal sequences must be recognized not only by SRP during the targeting step, but also at later stage(s) of the translocation process (Figure 10). Evidence for a second recognition event at the membrane was recently also provided by the observation that SRP-independent targeting required a functional signal sequence (Jungnickel and Rapoport, 1995).

Interestingly, the signal sequences analysed here display large variations in their capacity to interact with SRP and to promote vectorial transport. For example, the ovalbumin signal sequence binds SRP very efficiently, and its low translocation efficiency is only due to a poor recognition at the second step. The low translocation of mPAI-2 reflects a combination of reduced efficiencies during the two signal sequence recognition events. Another striking example is provided by the hA domain variants. The decreased affinity for SRP due to Asn8 accounts for its effect on translocation. In contrast, Asn14 has only a small negative effect on SRP binding, and its strong effect on translocation is mostly exerted during the second signal sequence recognition. The main determinant feature of signal sequences identified so far was hydrophobicity. Our results demonstrate that the contribution of individual residues on SRP binding and on translocation itself may be different. Thus, signal sequences may have a more complex structure than previously anticipated.

For all proteins analysed here, the decision to abort translocation must be made before Asn75, the second

N-glycosylation site of mPAI-2, becomes accessible to the oligosaccharide–transferase complex, since the cytosolic forms are not detectably glycosylated. The results of the flotation experiments suggest that the second recognition event can occur both before and after the formation of a stable ribosome–membrane complex, that is operationally defined by its resistance to high salt and EDTA treatment (Gilmore and Blobel, 1985; Connolly and Gilmore, 1986). Thus, translocation may be aborted at several time points during translocation. The movement of PAI-2 across the translocation channel could be bi-directional, and similar to that proposed for several other nascent chains (Garcia *et al.*, 1988; Ooi and Weiss, 1992); it may occur by a kinetic 'Brownian ratchet mechanism' analogous to that proposed by Simon *et al.* (1992).

The second recognition of signal sequences must be mediated by membrane components of the translocation machinery. Reconstitution experiments have shown that the Sec61p complex is necessary and sufficient for the second recognition of the preprolactin signal sequence (Jungnickel and Rapoport, 1995). In addition, genetic studies of SecY, the *Escherichia coli* homologue of the eukaryotic Sec61pα, have suggested that SecY interactions with signal sequences provide a proof-reading mechanism that can be altered to allow export of defective signal sequences (Derman *et al.*, 1993; Osborne and Silhavy, 1993).

It is generally believed that protein export and cytosolic accumulation are mutually exclusive. However, PAI-2 accumulates in both cellular compartments (Belin, 1993). Furthermore, the translocation efficiency of PAI-2 is different in different cell types and can be regulated during differentiation, under conditions where SRP levels do not change (D.Belin and K.Strub, unpublished results). An increased translocation efficiency of PAI-2 could result from improved targeting mediated by chaperones, which can promote protein translocation in yeast (Deshaies *et al.*, 1988). However, attempts to improve PAI-2 translocation *in vitro* in the presence of human Hsp70 or of the bacterial DnaK/DnaJ/GrpE chaperone were unsuccessfull (D.Belin, unpublished results). Thus, our data suggest that the bi-topological distribution of PAI-2 could be controlled at the membrane insertion/translocation step, during the second recognition of signal sequences. This may involve a functional modification of the Sec61p complex. Alternatively, the second recognition of signal sequences may also involve other component(s) of the translocon, such as a TRAM (Krieg *et al.*, 1989; Görlich *et al.*, 1992a), which can modulate translocation efficiency in reconstituted systems (Görlich and Rapoport, 1993), or TRAP, which can also establish physical contact with nascent chains (Hartmann *et al.*, 1993). Finally, it could reflect the activity of a mammalian homologue of the yeast Sec72p. This non-essential protein is involved in the recognition of a subset of signal sequences, including internal hydrophobic segments and signal sequences of low hydrophobicity (Feldheim and Schekman, 1994), two features of the PAI-2 and ovalbumin signal sequences.

In conclusion, signal sequences appear to be functionally more complex than previously recognised. They may have multiple features, designed to allow different interactions with the translocation machinery. This may ensure retention of proteins such as PAI-2 in the cytosol of mammalian cells, thereby allowing their secretion efficiency to be modulated.

Materials and methods

Plasmid construction and in vitro transcription with RNA polymerases

Plasmid pDB5702 contains a full-length hPAI-2 cDNA (Belin *et al.*, 1989). Plasmid pDB5907 contains a full-length mPAI-2 cDNA (EMBL Nucleic Acid Database AN: X16490) cloned in the sense orientation in pSP65 (Melton *et al.*, 1984). Plasmid pSP-PAI-2 contains the signal sequence of yeast pre-pro-α-factor fused in-frame to the PAI-2 coding region (Steven *et al.*, 1991). The N-terminal truncations were constructed as follows. pDB5911 contains a 1.8 Kbp *Pst*I–*Hin*dIII fragment of the mPAI-2 cDNA cloned between the cognate sites of pSP65. hA+hB deletion (pDB5908): the *Eco*RI–*Nco*I/Klenow fragment of the cDNA was cloned between the *Eco*RI and *Hin*cII sites of pDB5911; this removes amino acids 7–57 and introduces an Asp residue. hA deletion (pDB5910): an *Eco*RI–*Nco*I/Klenow fragment of the cDNA was first cloned between the *Eco*RI and *Sma*I sites of pUC19; a *Xba*I–*Bam*HI/Klenow fragment was then cloned between the *Xba*I and *Afl*II/Klenow sites of pDB5907; this removed amino acids 7–16, introducing a GlyVal linker (pDB5910). hB deletion (pDB5909): the *Eco*RI–*Afl*II/Klenow fragment of the cDNA was first cloned between the *Eco*RI and *Sma*I sites of pUC19; a *Xba*I–*Bam*HI/Klenow fragment was then cloned between the *Xba*I and *Hin*cII sites of pDB5911; this removed amino acids 18–57, introducing a GlyThrAsp linker. Plasmid cyc90 (Murray *et al.*, 1989) was the template of the sea urchin cyclin B mRNA. Plasmid pSPBP4 (Siegel and Walter, 1988b) was the template of bovine preprolactin mRNA. The second exon of the hen ovalbumin gene (AN: M34346), which encodes the N-terminal 56 residues of the protein, was PCR-amplified from genomic DNA (kind gift of Dr M.Ballivet). The resulting DNA fragment was subcloned between the *Bam*HI and *Pst*I sites of pDB5911. Plasmid pDB5511 encodes a chimeric protein (OVA-PAI2), whose N-terminal 56 ovalbumin amino acids are followed by amino acids 57–415 of mPAI-2.

For *in vitro* translation, mRNAs were synthesized from linearized plasmid templates in the presence of 500 μM cap analogue (Pharmacia), 50 μM GTP, 500 μM of ATP, CTP and UTP, and 3–10 μCi of [^{32}P]UTP (Belin *et al.*, 1989). Prior to translation, the RNAs were ethanol precipitated with 0.2 M KOAc pH 5.0 and resuspended in water (50 ng/μl).

Amino acid substitutions in the hA domain

Mutations in the hA domain were constructed using two degenerate oligonucleotides. P2A [5′CGGGATCCAACCATG(G/A)AA(G/A)AAC-TTTCCATGGC] was partially randomized at the first position of codons 2 and 3 to encode either the wild-type Glu or Lys residues. P2C [5′GCTCTAGACTTAAGGAGA(A/T)TGAGGGCAAACATGGTG(A/T)TTGCCATGGAAAG] was partially randomized at the second position of codon 8 and 14 to encode either the wild-type Asn or Ile residues. The oligonucleotides, which have 11 complementary residues at their 3′ ends, were annealed and elongated with the Klenow fragment of DNA polymerase I. After restriction with *Bam*HI and *Xba*I, the fragments were gel-purified and subcloned between the cognate sites of pUC19. The inserts were sequenced; only 10 of the 16 expected sequences were obtained (see Figure 6B and C), probably because of an uneven distribution of the randomized bases. The mutant hA fragments were isolated as *Eco*RI–*Xba*I fragments and cloned between the cognate sites of pDB5911, introducing a four amino acid SerArgValAsp linker between Lys17 and Leu58 (plasmids pDB5111–5114 and pDB5201–5206). In another project (D.Belin *et al.*, in preparation), hA domain mutants which had only an altered N-terminus but retained the two Asn residues have been generated. Three of these mutants were used to isolate *Eco*RI–*Afl*II fragments which were cloned between the cognate sites of pDB5111. Although the 5′ untranslated region in this group differs from that in the previous set of mutants, a sequence encoding a Lys2Glu3-Asn8-Asn14 type was present in both groups and both were translated indistinguishably (Figure 6, compare A, lanes 3 and 4 with C, lanes 5 and 6). All constructs were verified by sequencing the entire N-terminal region of mPAI-2 at least across the *Pst*I site encoding amino acids 58 and 59.

In vitro translation

Wheat-germ extracts were prepared as described (Dobberstein and Blobel, 1977) and used at 35 or 40% final concentration. Canine pancreas

microsomal membranes were either the kind gift of Dr Hwang [MIT, Cambridge (Hwang *et al.*, 1992)] or were purchased from Amersham, and were stored at –80°C in 0.25 M sucrose, 50 mM HEPES pH 7.4 and 2 mM DTT, and used at a final concentration of 0.24 Eq/10 µl reaction. SRP-depleted microsomal membranes were prepared according to Walter and Blobel (1983a). The [^{35}S]methionine was from Amersham. Synthetic PAI-2 mRNAs (5 µg/ml) were translated in the presence of 110 mM potassium acetate and 3.5 mM magnesium acetate, taking into account the salt concentrations contributed by the microsomes and/or SRP when present. TPCK-trypsin was from Sigma. SRP was purified as described (Walter and Blobel, 1983b) and its concentration determined by absorbance at 260 nm. Translations were usually performed for 90 min at 26°C. To measure the elongation arrest activity of SRP, the translation assays were incubated for 20 min; in all experiments, the concentrations of PAI-2 and cyclin nascent chains were similar within a factor of 3. The translation products were electrophoresed in 10 or 7.5% SDS–polyacrylamide gels, or in 15% gels to visualize nascent chains. The gels were fixed, dried and autoradiographed or exposed to a storage phosphor screen (Molecular Dynamics). The screen was scanned with a PhosphorImager (Molecular Dynamics), and the scans were analysed with the ImageQuant v3.22 software. Alternatively, appropriate exposures of the autoradiographs were scanned with a Densitometer (Molecular Dynamics), using the same software. Translocation efficiency was determined by measuring the intensities of the three *N*-glycosylation intermediates shown in the figures, and that of the unmodified protein; backgrounds were determined from parallel translations with microsomal membranes but without mRNA.

Flotation of the nascent chain–ribosome–membrane complexes

Plasmids digested with *Ssp*I (mPAI-2) or with *Pvu*II (preprolactin) were transcribed as described above. The truncated mRNAs, which encode nascent chains of 143 (mPAI-2) or 86 (preprolactin) amino acids, were translated for 20 min in the presence of SRP and microsomal membranes. After the addition of cycloheximide to 1 mM, 10 µl of translation reactions were mixed with 1 ml of ice-cold 80% sucrose in KHMD buffer (40 mM HEPES–KOH, pH 7.5, 100 mM KOAc, 5 mM MgOAc and 1 mM DTT). The mixtures were successively overlaid with 3 ml of 60% sucrose and with 1 ml of 10% sucrose in KHMD buffer brought to 0.5 M KOAc. After centrifugation for 16–18 h at 50 000 r.p.m. and 4°C in a SW55 rotor, 1 ml fractions, collected from the top of the gradients, were incubated for 15 min at room temperature with 10 µg of RNase A and 30 µg of BSA. Proteins were precipitated by the addition of 1 vol of 20% TCA, and collected by centrifugation for 30 min at 12 000 r.p.m. after 1 h at 0°C. Dried pellets were resuspended in sample buffer containing 0.15 M Tris–HCl pH 9.5 and boiled for 2 min prior to electrophoresis.

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